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GLUTAMATE AND BRAIN GLUTAMINASES IN DRUG ADDICTION

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Abstract:	<p>Glutamate is the principal excitatory neurotransmitter in the central nervous system and its actions are related to the behavioral effects of psychostimulant drugs. In the last two decades, basic neuroscience research and preclinical studies with animal models are suggesting a critical role for glutamate transmission in drug reward, reinforcement, and relapse. Although most of the interest has been centered in post-synaptic glutamate receptors, the presynaptic synthesis of glutamate through brain glutaminases may also contribute to imbalances in glutamate homeostasis, a key feature of the glutamatergic hypothesis of addiction. Glutaminases are the main glutamate-producing enzymes in brain and dysregulation of their function have been associated with neurodegenerative diseases however, the possible implication of these enzymes in drug addiction remains largely unknown. This mini-review focuses on brain glutaminase isozymes and their alterations by in vivo exposure to drugs of abuse, which are discussed in the context of the glutamate homeostasis theory of addiction.</p>	

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GLUTAMATE AND BRAIN GLUTAMINASES IN DRUG ADDICTION

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Abbreviations: DA, dopamine; eCB, endocannabinoids; GA, glutaminase; GAB, *Gls2*-encoded
long glutaminase protein variant; GAC, *Gls*-encoded short glutaminase protein variant; *Gls*,
glutaminase gene encoding the kidney-type isoforms; *Gls2*, glutaminase gene encoding the
liver-type isoforms; KGA, *Gls*-encoded long glutaminase protein variant; LGA, *Gls2*-encoded
short glutaminase protein variant; LPA, lysophosphatidic acid; MSN, medium spiny neurons;
NrCAM, neuron-glia related cell adhesion molecule; PLG, prolyl-leucyl-glycinamide.

Keywords: glutaminase; cocaine; drugs of abuse; glutamate; glutamatergic neuroadaptations;
synaptic plasticity.

Abstract

Glutamate is the principal excitatory neurotransmitter in the central nervous system and its actions are related to the behavioral effects of psychostimulant drugs. In the last two decades, basic neuroscience research and preclinical studies with animal models are suggesting a critical role for glutamate transmission in drug reward, reinforcement, and relapse. Although most of the interest has been centered in post-synaptic glutamate receptors, the presynaptic synthesis of glutamate through brain glutaminases may also contribute to imbalances in glutamate homeostasis, a key feature of the glutamatergic hypothesis of addiction. Glutaminases are the main glutamate-producing enzymes in brain and dysregulation of their function have been associated with neurodegenerative diseases and neurological disorders; however, the possible implication of these enzymes in drug addiction remains largely unknown. This mini-review focuses on brain glutaminase isozymes and their alterations by *in vivo* exposure to drugs of abuse, which are discussed in the context of the glutamate homeostasis theory of addiction. Recent findings from mouse models have shown that drugs induce changes in the expression profiles of key glutamatergic transmission genes, although the molecular mechanisms that regulate drug-induced neuronal sensitization and behavioral plasticity are not clear.

Brain glutaminases

The mammalian glutaminase (GA; EC 3.5.1.2) family members are encoded by two paralogous genes, *Gls* and *Gls2*, presumably derived from a common ancestral gene by duplication and divergent evolution [1,2]. In humans, the *GLS* gene is located on chromosome 2 and encodes GA isozymes classically referred to as kidney-type (K-type), while *GLS2* is located on chromosome 12 and codes for liver-type (L-type) isozymes [3]. Orthologous genes have been described in rat [4] and mouse [5]. The human *GLS* gene spans 82 kb and is split into 19 exons [1]. Two different transcripts arise from this gene: the KGA transcript, originally found in kidney, composed by 18 exons and formed by joining exons 1 to 14 and 16 to 19, and the GAC transcript which appears by alternative splicing and uses only the first 15 exons [1,6]. The human *GLS2* gene has a length of approximately 18 kb and is split into 18 exons [2]. Two transcripts have been identified from the mammalian *Gls2* gene: the canonical long transcript termed GAB, formed by joining all 18 exons of the gene [2], and the short transcript LGA that lacks exon 1 and was originally identified in rat liver [7].

KGA was originally thought as the only glutaminase present in brain [8,9]. Nowadays, the pattern of GA expression in mammalian tissues has been shown to be considerably more complex. Concretely, we first reported that human brain expresses GLS2 transcripts, in addition to the KGA isoform; these transcripts were ubiquitously expressed in brain regions with the strongest signal appearing in cerebral cortex [3,10]. Shortly after, Northern analysis and immunocytochemistry in brain of diverse mammalian species (human, monkey, rat, cow, mouse and rabbit) confirmed simultaneous expression of GLS (KGA) and GLS2 (GAB and/or LGA) isoenzymes; interestingly, both isoforms colocalize in numerous cells throughout the brain [11]. Furthermore, the existence of alternative transcripts of the *Gls2* gene was recently demonstrated in brain (and liver) of three mammalian species: human, rat and mouse [12]. Two GLS2 transcript variants showing alternative first exons were amplified: the long GAB transcript, previously cloned from ZR-75 breast cancer cells [10], and the short LGA transcript first characterized in rat liver [4,7]. The LGA variant appears by use of an alternative transcription

start site (TSS): both the TSS and one LGA-specific alternative promoter are located in the first intron of the *GLS2* gene, which is located 7 kb away from the canonical promoter of the GAB isoform [12]. Quantitative measurements of GLS2 transcript variants by real-time RT-PCR demonstrated that the ratio of the two products varied very widely between tissues and species [12]. Thus, while LGA was slightly more abundant than GAB in mouse brain, the opposite was found in rat brain where GAB mRNA level was fourfold the amount of LGA. In human brain tissue and cultured neurons and astrocytes, KGA and GAC transcripts showed similar expression levels [13]. In mammalian brain, evidence supporting expression, at the protein level, of the four different GA mRNAs (KGA, GAC, GAB and LGA) has been obtained for all of them except for GAC isozyme, by employing isoform-specific antibodies against whole proteins, truncated proteins and peptide sequences (reviewed in [14]).

Glutamine is an important precursor for glutamate synthesis in the brain through GA reaction [15,16]. GA is both an important contributor to the transmitter pools of glutamate [16] and the main glutamine-utilizing enzyme in neurons [15]. For this reason, GA is regarded as the main producer enzyme of presynaptic glutamate in the brain. Various regions specifically involved in glutamatergic transmission, such as the cerebral cortex, hippocampus, striatum and cerebellum, were emphasized as those presenting the more intense GA immunolabeling [17]. The identification of the function of each GA isozyme is essential for understanding their roles in cerebral function. Interestingly, GLS2 isoenzymes were not only expressed in neuronal cells, but also were found in astrocytes [18,19]. Besides its roles in neurotransmission and metabolism, glutamate biosynthesis has been shown to regulate synaptic plasticity, neurogenesis, neurite outgrowth, and neuron survival in the developing and adult mammalian nervous system [20]. Of note, altered glutamate/glutamine homeostasis has been also involved in the pathogenesis of neuropsychiatric diseases [21]. However, the role of presynaptic glutamate biosynthesis in the addictive process to drugs of abuse has been considerably less studied.

Glutamatergic theory of addiction

The brain reward circuitry includes midbrain dopaminergic neurons in the ventral tegmental area (VTA) and the brain regions that are innervated by projections from the VTA, mainly the nucleus accumbens (NAc), the prefrontal cortex (PFC), the amygdala, and the hippocampus [22], as well as descending feedback projections. This mesocorticolimbic circuit is responsible for reward and reinforcement processing and for changing behaviors in response to motivations, emotions, affective information and environmental cues that can acquire secondary reinforcing properties when paired to repeated drug exposure [23]. The feedback descending projections is composed by the cortical projections to the extended amygdala (the amygdala, the NAc and the septum, all innervated by the VTA dopaminergic neurons), and the motor subcircuit, that includes prefrontal and motor cortex projections to the dorsal striatum and that is innervated by the substantia nigra dopaminergic neurons [24]. Both cortical and limbic nuclei send major glutamatergic projections to the NAc, which is subdivided into the shell and core subregions; the NAc sends GABAergic projections to the VTA/substantia nigra which, in turn, sends GABAergic projections to the medial dorsal thalamus (mDT); finally, glutamatergic projections from the mDT to the mPFC close the limbic circuit [25]. Thus, the NAc represents a functional interface between limbic and motor systems and receives a substantial input of glutamatergic afferents that are regulated by dopaminergic projections from the VTA.

Sound evidences obtained from the 1970s have now generated a general consensus in the field of drug addiction pointing to the dopamine (DA) system as the initial target of all addictive drugs [26]. Drugs of abuse interact with the mesocorticolimbic nuclei, increasing DA levels in this system [27]. However, the increase in DA levels occurs by different mechanisms and seems to be drug-specific [28]. Thus, the dopamine hypothesis of addiction has established that the strong activation of mesolimbic dopamine is a pervasive and defining feature of all addictive drugs. However, while the requirement for increased DA may explain the acute reinforcing effects of addictive drugs, it does not provide an explanation for long-lasting behavioral abnormalities, like craving and relapse, which are key features of addiction seen even when drugs have been cleared from the body and DA levels returned to normal. Therefore,

changes in DA signaling cannot be the cause of adaptive behavior which manifest long after the drugs have been cleared from the brain (reviewed in [26, 29]). Accordingly, drug-evoked changes in concrete neurotransmission circuits of the mesocorticolimbic region were suggested to play a causal role in the pathological behavior of addictive patients [30]. Although many types of synapses can be modified by addictive drugs, the focus has been centered on various forms of synaptic plasticity exhibited by glutamatergic transmission in the circuits of the mesolimbic system [31]. Preclinical evidences obtained from studies with rodent models of addiction put forward the glutamate homeostasis hypothesis of addiction, which essentially proposes the existence of neuroplasticity changes elicited by an imbalance between synaptic and non-synaptic glutamate in corticostriatal circuits that undermines connections between PFC and NAc, yielding a lower capacity to inhibit drug-seeking behavior and increasing vulnerability to relapse [24]. In fact, two types of effects on glutamatergic projections to the NAc are now being considered in addiction: slow and enduring changes elicited by the chronic drug use [32], which are a major regulator of the addictive behavior and yield persistent neuroadaptations recently coined as constitutive synaptic potentiation [33], and rapid transient increases in synaptic strength of glutamatergic synapses between PFC afferents in medium spiny neurons (MSN) of the NAc core, termed transient synaptic potentiation, which reflect dynamic plasticity changes associated with drug seeking neuropathology and relapse [32, 33]. The constitutive adaptations are seen after extended withdrawal from chronic drug use, while transient changes are produced during cue- or drug-induced reinstatement of drug seeking [33].

Glutamate is the main excitatory neurotransmitter in the mammalian central nervous system [34]. Furthermore, over 90% of the whole synapses are glutamatergic [35] and the Glu/Gln cycle between neurons and astrocytes have been postulated as a central regulatory motif for glutamate (and glutamine) homeostasis in brain [36,37]. Glutamate homeostasis also involves the regulation of extracellular glutamate levels in the synaptic and perisynaptic environments; interestingly, these glutamate pools modulate synaptic strength and plasticity through interactions with ionotropic and metabotropic glutamate receptors and are essentially

modified by most drugs of abuse (Fig. 1). Thus, in acute and chronic treatments with cocaine, heroin and nicotine, as well as in reinstatement by acute drug intake or environmental cues associated to drug exposure, marked alterations of extracellular glutamate levels occurs in the NAc, striatum, PFC and VTA regions [28,38]. These changes can be of different magnitude and range (increments or reductions) depending of the type of drug treatment and withdrawal schedule. In this regard, one of the best characterized glutamatergic transmission changes has been reported in the NAc region. Acute administration of cocaine has a null or negligible effect on extracellular glutamate levels in the NAc [39]; in sharp contrast, withdrawal from chronic exposure reduces basal extracellular glutamate levels in the NAc [25]. Interestingly, a challenge injection of cocaine during extended withdrawal decreased presynaptic glutamate content in the NAc core but not in the NAc shell, suggesting different neuroadaptations in both subregions [25 and references therein]. In fact, two parallel pathways in the control of drug craving have been previously proposed: the prelimbic cortex to NAc core pathway, which promotes drug-seeking behavior, and the infralimbic cortex to NAc shell pathway, which is responsible for the extinction of drug-seeking behavior [40].

The impaired glutamate homeostasis in the corticostriatal circuitry can be explained by drug-induced presynaptic and glial adaptations giving rise to maladaptive synaptic plasticity. For example, the decrease in basal accumbal glutamate during withdrawal from chronic cocaine exposure, mentioned above, resulted from reduced activity of the cystine-glutamate antiporter. The main source of extracellular glutamate in this region came from the activity of the astrocytic Xc^- cystine-glutamate antiporter, which exchanges extracellular cystine by intracellular glutamate with a 1:1 stoichiometry [41]. Repeated cocaine administration downregulates the glial Xc^- exchanger leading to the reduced basal extracellular glutamate levels in the NAc, frequently observed in animal models of addiction [24,41] (Fig. 1). Therefore, the NAc core synapse shows a fall in the pool of non-synaptic glutamate at the extracellular space following both chronic administration and withdrawal from addictive drugs, like cocaine and methamphetamine [42]. Of note, activating the Xc^- antiporter with N-

acetylcysteine restores basal extracellular levels of glutamate and prevents the reinstatement of cocaine and heroin seeking [24,41]. The decreased extrasynaptic glutamate concentration induces an enhanced release of synaptic glutamate in the NAc core through inactivation of presynaptic inhibitory metabotropic glutamate mGluR2/3 receptors [32] (Fig. 1). The reduction in mGluR2/3 autoreceptor signaling arises by downregulation of protein expression, as well as by upregulation of activator of G protein signaling 3 (AGS3) which inactivates $G_{i\alpha}$ subunit [33]. The lack of tone on metabotropic mGluR2/3 receptors potentiates the synaptic release of glutamate. Accordingly, direct agonist stimulation of mGluR2/3 inhibits cocaine and heroin seeking [24]. The overflow of synaptic glutamate was attenuated by pharmacological inactivation of the mPFC [43], whereas administration of AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor antagonists into the NAc blocked the reinstatement of cocaine seeking [44]. Taken together, these findings strongly suggest that activation of glutamatergic projections from the mPFC to the NAc, along with stimulation of AMPA receptors, play a critical role in cocaine seeking, in agreement with brain imaging studies of human cocaine addicts showing metabolic activation of the mPFC during cocaine craving [45].

In addition, another drug-induced glial adaptation essentially contributes to the heightened synaptic glutamate levels in the NAc: the abnormally high amount of glutamate released in the synaptic cleft is not efficiently removed, because the glial excitatory amino acid transporter GLT-1 (EAAT2) is also downregulated in the NAc following chronic exposure to cocaine, nicotine, ethanol, and heroin (extended withdrawal from cocaine causes further silencing) [42]. Therefore, synaptic glutamate levels are increased owing to enhanced release and to reduced elimination from the extracellular space. Interestingly, the enhanced synaptic glutamate release in the NAc was also observed during the reinstatement of extinguished drug seeking, provoked by exposure to drug-associated cues, stress or drug itself. Thus, enhanced levels of extracellular NAc glutamate due to an increased presynaptic release were found during the reinstatement of cocaine, nicotine, methamphetamine, heroin and alcohol [24,42,46-48]. These excessive glutamate amounts and overflow of synaptic glutamate during reinstatement

may explain how drug-associated cues provoke such a strong behavioral effect. This glutamate overflow causes spillover and additional activation of synaptic (AMPA, NMDA (N-methyl-D-aspartate) and nonsynaptic glutamate receptors, particularly ionotropic GluN2B containing NMDA receptors and metabotropic mGluR5 receptors [33,42].

A final consequence of drug-induced changes in NAc glutamatergic transmission is the postsynaptic remodeling that induces structural plasticity, giving rise to constitutive synaptic potentiation. Glutamatergic synaptic plasticity has been described in dopamine D1R neurons and GABAergic MSN during prolonged drug exposure, withdrawal and cue-induced relapse to drug seeking (see [28] and [49] for a review). For example, during cocaine, nicotine and heroin-reinstatement there were increases in dendritic spine head diameter and AMPA receptor currents due to a shift in the AMPA/NMDA ratio [32,46,50]; these are typical characteristics of a long-term potentiation (LTP) state (Fig. 1). Furthermore, it is well known that the number of dendritic spines on NAc MSN increases after repeated exposure to cocaine, amphetamine and nicotine [51]. Many studies have demonstrated the relevance of dendritic spine morphology in hippocampus and cerebral cortex; concretely, their size and shape correlate with different forms of synaptic plasticity such as LTP and long-term depression (LTD) [52]. Furthermore, stimulants like cocaine, amphetamine and methylphenidate consistently increase dendritic complexity and spine density of MSN in the NAc, dopaminergic neurons in the VTA and pyramidal neurons of the mPFC [49]. Of note, the drug-evoked synaptic plasticity in the NAc has been recently proposed as a causal mechanism controlling cocaine relapse [53].

On the other hand, a neuroproteomic study in rats with self-administration of cocaine found significant changes in proteins related to synapse function and neuronal remodeling; curiously, a number of them remained altered even after long periods (100 days) of abstinence [54]. Moreover, results from proteomic studies of the NAc in monkeys following chronic cocaine self-administration found quantitative differences in proteins involved in cytoskeleton structure and remodeling, mitochondrial function, energy metabolism and cell signaling [55]. A significant number of these proteins were either directly or indirectly related to the

hyperglutamatergic state identified in rhesus monkeys self-administered with cocaine and also in cocaine overdose victims [56]. In addition to the Xc^- exchanger, extracellular glutamate is derived from vesicular synaptic and non-vesicular glial release. While the expression of the glial GLT-1 transporter has been shown to be diminished following exposure to drugs, scarce information was available regarding the effect of drug of abuse on the regulation of presynaptic glutamate synthesis, via glutaminase, and its relevance in the activation of glutamatergic transmission and synaptic plasticity associated with craving and relapse after drug withdrawal.

Glutaminases and drug addiction

The regulation of presynaptic glutamate synthesis and release may also contribute to drug craving and relapse to drug seeking. Changes in neurotransmitter release are thought to underlie the decreased inhibitory modulation of pyramidal neurons in the PFC during early withdrawal from repeated cocaine exposure; however, this attenuation in inhibitory transmission seems to be shifted at the glutamate receptor level during more prolonged withdrawal [57]. Therefore, we aimed to ascertain whether GA isoforms would also contribute to trigger pathophysiological release of synaptic glutamate, a frequent alteration observed in animal models of cocaine addiction. While glutamate receptors, namely metabotropic, and their ligands are well documented to be potential therapeutic targets for addiction [26,42], GA had not been investigated in almost two decades since it was studied for the physical dependence upon and tolerance to opiates [58]. It was previously shown that subchronic and acute administration of GA inhibitor prolyl-leucyl-glycinamide (PLG) modulates morphine physical dependence by inhibition of glutamate production, an effect consistent with the hypothesis of dependence to opiates which states that these drugs inhibit GA in the nerve ends [59]. Interestingly, acute administration of inhibitors of glutamate release, such as tizanidine, attenuated the abstinence syndrome to opiates, as did PLG; this effect was explained by the lesser release of glutamate and, concomitantly, the lesser activation of receptors [58].

More recently, the GA inhibitor PLG was employed in studies dealing with the role of neuron-glial related cell adhesion molecule (NrCAM) in addiction vulnerability for polysubstance abuse. NrCAM is a regulator of axon growth, dendritic spine remodeling and hippocampus development, and has been associated with drug abuse vulnerability in humans and mice [60,61]. *Nrcam* knockout (KO) mice were generated in order to gain insight into NrCAM protein involvement in addiction; interestingly, expression of *Gls*, a key glutamatergic gene coding for K-type GA isoforms, was significantly downregulated in PFC and ventral midbrain regions in heterozygous mice compared with wild-type littermates. This unexpected finding was also confirmed in T98G human glioblastoma cells treated with siRNA against the *NRCAM* gene: characterization of their transcriptome by microarray analysis revealed that *GLS* was downregulated more than 50% in cells with silenced expression of *NrCAM* [62]. Decreased NrCAM expression in brain is protective against addiction vulnerability: *Nrcam* KO mice do not develop conditioned place preferences for morphine, cocaine or amphetamine [60]. Similar phenotypes were produced in mice treated with the GA inhibitor PLG [62], in accordance with this effect being mediated by altered presynaptic glutamate synthesis. Hence, these authors conclude that NrCAM regulates addiction-related behaviors at least partially through regulation of GA (*Gls*) expression, reinforcing the glutamate homeostasis theory of addiction.

The genomic dysfunctions induced by acute and chronic cocaine administration in the glutamatergic system were recently characterized by analyzing the expression of genes involved in the biosynthesis and transport of glutamate in mouse brain. We assessed the short/long-term mRNA modifications caused by differences in acute treatment (vehicle or cocaine for 1 day), chronic pretreatment (conditioning with vehicle or cocaine for 5 days) and its interaction (acute treatment x chronic pretreatment) (Fig. 2). In this way, we had four experimental groups: animals conditioned with cocaine (20 mg/Kg) and administered with vehicle or cocaine (10 mg/Kg), and animals conditioned with vehicle and treated with vehicle or cocaine (10 mg/Kg) (Fig. 2). The expression profiles of genes coding for the neuronal excitatory amino acid carrier 1 (EAAC1) and enzymes of glutamate synthesis (KGA and GLS2 GA isoforms) in striatum,

hippocampus, prefrontal cortex and cerebellum of treated-mice were analyzed [63]. The results showed that acute administration of cocaine resulted in a dose-dependent increase of locomotor activity, while chronic treatment induced conditioned locomotion and behavioral sensitization. Changes in mRNA levels of GA isoforms and neuronal glutamate transporter EAAC1 (EAAT3) were observed: the strongest effects were mostly seen after acute cocaine challenge. Regarding the EAAC1 carrier in striatum, our data showed statistically significant decreased expression after acute cocaine, in both control (V-C group) and cocaine-sensitized animals (C-C group) (Fig. 2), suggesting that this neuronal Glu transporter plays also a role in the neuroadaptations to cocaine. Thus, its downregulation might also contribute to the known increase of synaptic accumbal glutamate and glutamatergic signaling, through regulation of pre-synaptically released glutamate during withdrawal from cocaine exposure [63]. In case of the EAAC1 transporter mRNA expression in cerebellum, we observed a chronic effect induced by chronic pretreatment, but not by acute treatment (Fig. 2). This enhancement in the levels of Glu transporter mRNA could be due to initial chronic pretreatment with cocaine in Purkinje cells where the EAAC1 transporter is especially abundant. The remaining regions studied, hippocampus and PFC, did not present differences in expression levels [63].

KGA mRNA expression is activated by chronic-cocaine pretreatment in striatum, but did not show significant differences in acute treatment (Fig. 3). Repeated administration of cocaine produced long-term overstimulation throughout the increased neuronal excitability of glutamatergic striatal neurons. This neural excitation may be caused by an enhanced KGA enzymatic activity related to an augmented locomotion induced by cocaine conditioning and is consistent with the glutamate homeostasis hypothesis of addiction. Instead, KGA mRNA expression in hippocampus is inhibited by both acute- and chronic-cocaine treatments (Fig. 3) [63-64]; however, since non-statistically significant differences were found between both groups, this reduced expression could not be ascribed to cocaine sensitization. The decrement of hippocampal KGA gene expression can be linked to the lower gene expressions of metabotropic mGluR3 and GluR5 glutamate receptors and GluN1, GluN2A, GluN2B and GluN2C NMDA

receptor subunits following acute cocaine administration [64]. Interestingly, locomotor sensitization to cocaine was specifically associated with an increased gene expression of GluN1 and GluN2C NMDA receptor subunits [64].

On the other hand, GLS2 mRNA expression is strongly activated by cocaine in the striatal region after chronic injection protocols (Fig. 3); we found an interaction between acute and chronic treatments which suggest this effect might be due to robust cocaine-induced neuronal sensitization. In the PFC, GLS2 mRNA increases in acute cocaine priming, as well as in acute cocaine/sensitized mice (Fig. 3) [63,65], while its expression levels remain essentially unchanged in hippocampus and cerebellum [63,64] (although in this last region, a significant increase was detected for the C-C group vs the V-V group [66]). It is noteworthy that both GA isoforms showed a strikingly different pattern in the cocaine addiction paradigm described above. This markedly distinct regulation of GA isoforms by cocaine would be related to their different roles, recently unveiled for GLS and GLS2 isoenzymes. Thus, novel GA tasks include neuronal development and maturation for KGA isozyme, and transcriptional regulation, associated with its nuclear localization, for GLS2 isoforms [14]. Nevertheless, in terms of absolute quantitative levels, KGA mRNA levels are much more abundant than GLS2 (L-type) GA in striatum, hippocampus, prefrontal cortex and cerebellum [63], as well as in rat and mouse whole brain [12]. These data are consistent with our previous results at the protein level, showing that KGA immunostaining was more pronounced in the mammalian brain than GLS2 [11,18,19].

Total GA activity is decreased after repeated exposure (C-V group) to cocaine using a dose regimen that induces behavioural sensitization to this psychostimulant: one priming injection after chronic administration of cocaine (C-C group) also elicited a decrease in GA activity in both the dorsal striatum and the PFC vs V-V or V-C groups (Fig. 3). After chronic cocaine pretreatment, neither a vehicle injection nor a challenge with a cocaine injection was able to recover the GA activity values to those shown by acute control (V-V) or acute cocaine-treated (V-C) animals (Fig. 3). Cocaine-induced sensitization is associated to enhance glutamate

transmission in both brain areas owing to dynamic changes in both glutamate release and glutamate receptor signaling, as we detailed in the previous section. In this context, the decrease in GA activity may be either a counter-regulatory inhibition to compensate the enhanced glutamate output or be related to the fact that withdrawal from repeated exposure to cocaine reduces basal extracellular glutamate levels in the basal ganglia [25]. Since we observed a reduced GA activity 5 days after cessation of repeated cocaine exposure, this explanation seems to be plausible. Overall, these results set in place GA as a relevant protein involved in the neuroadaptations associated to repeated cocaine exposure. Main changes were observed on the prefrontal cortex-dorsal striatum circuit, which is the anatomical site for the neuroplasticity events that underlie the cocaine-induced behavioral sensitization. The results represent a molecular basis to explain the cocaine-induced and region-specific alterations of extracellular glutamate in the mesocorticolimbic circuit. Moreover, cocaine affects glutamatergic enzymes and transporter with sharply different patterns specific for the brain area under study, inducing differential long-lasting effects in striatum glutamate receptors. Furthermore, cocaine may exert different roles in pre/postsynaptic neuronal membrane by regulation of glutamate synthesis and transport depending on acute or chronic administration regimen.

Involvement of endocannabinoids and biolipids

Finally, the role of endocannabinoids (eCB) and bioactive lipids, as modulatory systems of glutamatergic neurotransmission in cerebral areas involved in drug addiction, also deserves some comments. Glutamatergic transmission is retrogradely modulated by eCB through the stimulation of CB1 receptors located in the glutamatergic and GABAergic axon terminals and preterminals of the brain [67]. Both acute and repeated cocaine administration modulate the expression of the endocannabinoid signaling machinery in PFC, hippocampus and cerebellum. For example, after chronic cocaine exposure there is a decreased expression of the eCB-degradation enzyme fatty acid amino hydrolase (FAAH) and eCB-producer enzyme diacylglycerol lipase β (DAGL β) in mouse cerebellum, suggesting a down-regulation of 2-arachidonylglycerol (2-AG) production, which is responsible for inhibiting the excitatory

glutamatergic inputs of granule cells [66]. The lack of eCB-mediated inhibition might be partially responsible for a hyperactivated glutamatergic state in cerebellum (increased expression of GLS2 after chronic cocaine pretreatment), similar to the well-described hyperglutamatergic state detected in the NAc with prolonged cocaine action [24,33]. Of note, results obtained in mouse hippocampus and PFC demonstrated that chronic exposure to cocaine resulted in a down-regulation of the endocannabinoid-production/degradation enzyme ratios, suggesting a reduced endocannabinoid tone, along with an upregulation of CB1 receptors [64,65]. This over-expression of the CB1 receptor could be a compensatory response to counteract the low availability of retrograde endocannabinoid inhibitory signals. In conclusion, these results indicate that cocaine administration induces a reduced hippocampal and PFC endocannabinoid tone which could be linked to a hyperactive glutamatergic signaling.

On the other hand, it is well documented the role of lysophosphatidic acids (LPA) as mediators of learning, emotional behavior and synapse formation through modulation of dendritic spine dynamics. Although many types of LPA receptors have been detected in brain, LPA₁ is abundantly expressed and considered the most prevalent receptor type in both embryonic and adult brains [68]. Genetic silencing of LPA₁ in mice causes deficiencies in spatial memory retention and abnormal use of searching orientation strategies [69], defective working and reference memory independently of exploratory and emotional impairments attributed to hippocampal malfunction [70]. With regard to drugs of abuse, altered expression of hippocampal metabotropic mGluR3 glutamate receptors and the GLUR1 subunit of AMPA receptor were reported in KOLPA₁ mice, associated to an attenuated conditioned locomotion (CL) response after cocaine conditioning [71]. Furthermore, genetic deletion and pharmacological blockade of LPA₁ receptor results in increased alcohol consumption in mice, suggesting a relevant role for the LPA/LPA₁ signaling pathway in alcoholism [72]. Interestingly, KOLPA₁ mice show a strong downregulation of KGA protein expression mostly in cerebral cortex and hippocampus (Authors, manuscript in preparation). The KGA protein deficit might be linked to the altered morphology of hippocampal dendritic spines shown by

KOLPA₁ mice: these animals presented a less mature, filopodia-like, phenotype as compared with wild-type littermates. Several protein markers involved in spine maturation were then analyzed: both protein expression level and activity of matrix-metalloproteinase 9 (MMP-9) were significantly reduced in KOLPA₁ mice (Authors, manuscript in preparation). In conclusion, LPA signalling through LPA₁ strongly influences expression of the main isozyme of glutamate biosynthesis (KGA) and has repercussions on dendritic spines architecture, which might partially explain the cognitive and learning defects shown by these animals. In this regard, it is worth mentioning that synaptic plasticity mediating cocaine relapse also requires matrix metalloproteinases [73]; thus, although it is not currently understood how long-lasting synaptic potentiation at glutamatergic synapses is required for relapse to drug-seeking, re-shaping of dendritic spine morphology through LPA₁ signaling (and/or brain GA) appears as a plausible mechanism supporting long-term corticostriatal glutamatergic plasticity underlying drug relapse and drug-seeking behaviors.

Future directions

Sound experimental evidences to confirm the causal relationship between altered synaptic plasticity in glutamatergic synapses of the corticolimbic system and the drug-seeking behavior and relapse are still lacking. In this regard, novel animal models of addiction and the new field of optogenetics, where the composition and number of glutamate receptors may be controlled by light pulses, can give essential information that will be later validated in human studies. Synaptic glutamate spillover during drug seeking is a potential pharmacotherapeutic target that seems to appear ubiquitously across drug classes. Therefore, molecules targeting key proteins involved in this glutamatergic neuroadaptation have demonstrated a good response by reducing drug seeking in animal models; for example, N-acetylcysteine and ceftriaxone through activation of the downregulated GLT-1 (EAAT2) transporter, and mGluR2/3 agonists or AGS3 peptide inhibitors via activation of mGluR2/3 receptor signaling [33].

Elucidation of the molecular mechanisms associated with drug-induced neuroadaptations will certainly improve the few effective therapeutic options available to date. The picture of proteins involved in the control of glutamate homeostasis seems not yet fully unveiled; it should include not only postsynaptic receptors, but also proteins participating in the presynaptic synthesis of glutamate and its release into the synaptic cleft. In this sense, brain glutaminases should be added to the list of novel candidates for the pharmacotherapy of drug craving and relapse. However, the lack of potent and specific GA inhibitors with good brain penetrant behavior has precluded so far testing GA inhibition in the pharmacotherapy of many neurological alterations and diseases. In spite of this limitation, mouse models with defective expression of brain GA isoforms have recently demonstrated a strong potential for evaluation of GA inhibition therapy in the pharmacotherapy of neuropsychiatric disorders. Remarkably, mice with only one functional *Gls* allele (and with a concomitant reduction in GA activity) showed a schizophrenia resilience phenotype [74], while at the same time having a benign side effect profile [75]. Therefore, genetic pharmacotherapy appears as a valid alternative for drug development strategy against neurological disorders, including addiction, while new and more effective GA inhibitors are developed.

Finally, apart from the classic route of glutamate synthesis, release and clearance at the synapsis, other neurotransmitters, such as endocannabinoids and neuroactive lipids, can modulate glutamatergic transmission and may become relevant pieces in the puzzle of addictive behaviors to drugs of abuse.

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FIGURE LEGENDS

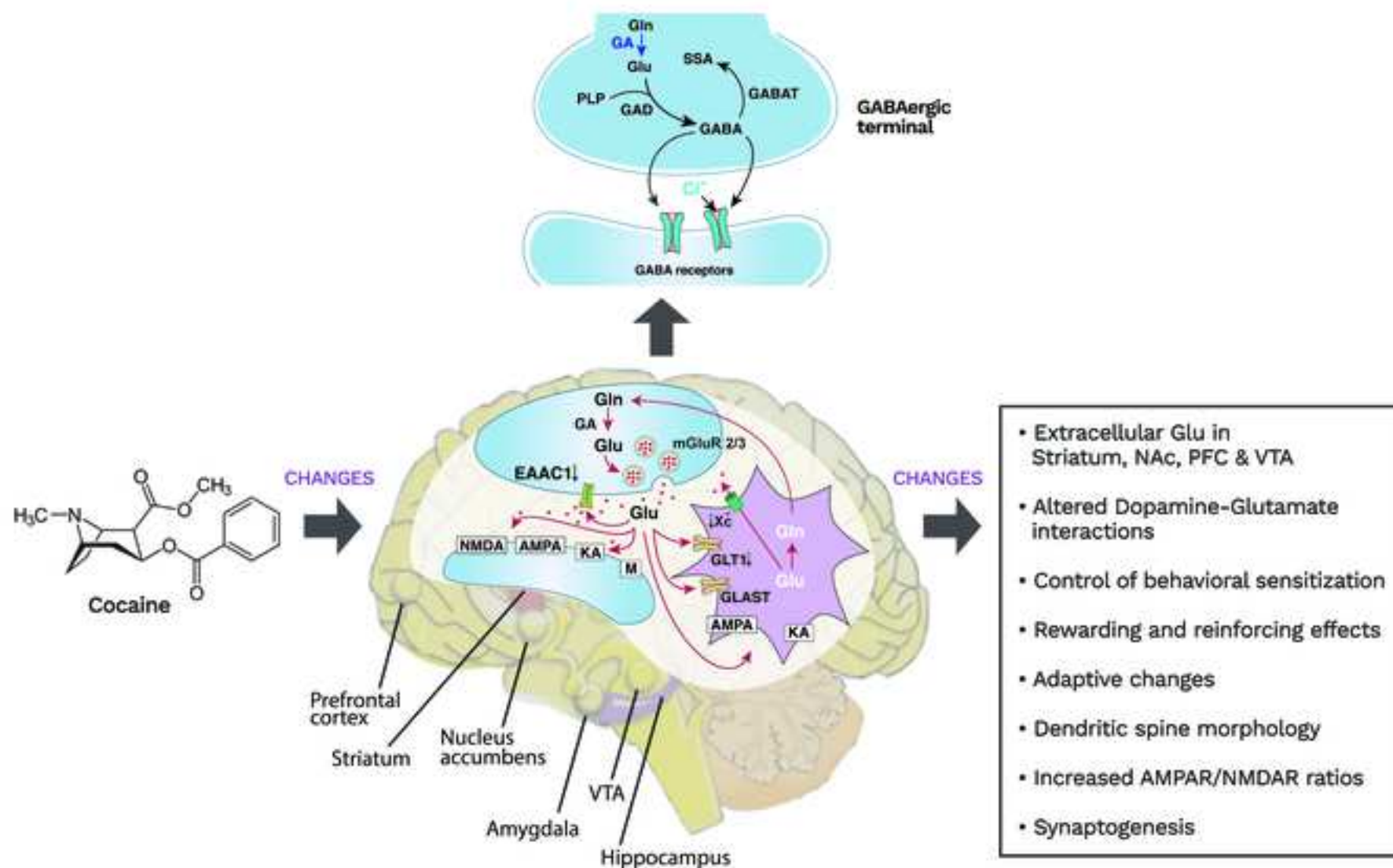
Figure 1. Altered glutamatergic transmission in cocaine addiction. This scheme illustrates the regions involved in the corticostriatal brain reward circuitry. The main glutamatergic neuroadaptations (changes in glutamatergic synapses) in cerebral regions closely connected to the addictive behavior to stimulant drugs are indicated. Although most of these variations were characterized for cocaine, some of them, as explained in the article, are also shared by other stimulant drugs such as heroin, nicotine, morphine and alcohol. Relevant adaptations include altered extracellular glutamate levels (increases or reductions depending on the region and type of addiction paradigm), changes in the expression of neuronal and glial glutamate transporters and postsynaptic plasticity (variations in the AMPAR/NMDAR ratio, dendritic spine morphology and/or number and activity of MMPs). Arrows indicate relevant drug-induced synaptic changes that promote neuroadaptations in the NAc leading to increased synaptic strength. The potentiation of GABAergic synapses in the VTA region is also indicated at the top of the figure. GA, glutaminase; GABAT, GABA transaminase; SSA, succinic semialdehyde; GAD, glutamate decarboxylase; PLP, pyridoxal 5'-phosphate; KA, kainate receptors; M, metabotropic glutamate receptors.

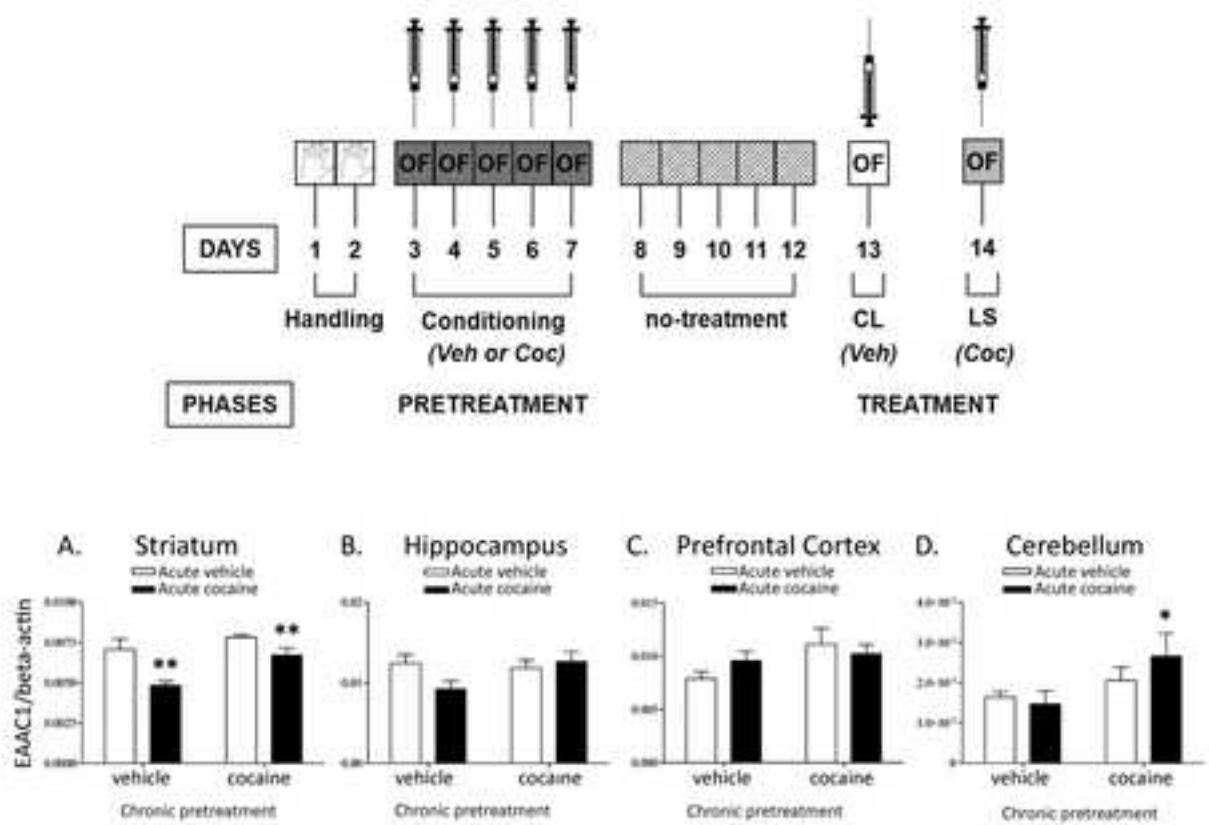
Figure 2. Protocol employed to evaluate cocaine-induced conditioned locomotion (CL), locomotor sensitization (LS) and gene expression modifications of the glutamate carrier EAAC1. **Top panel:** schematic representation of the consecutive 5-phase-paradigm for acquisition and expression of cocaine-induced sensitization. Following two days of handling, mice were daily administrated i.p. with vehicle or cocaine (20 mg/Kg) during five consecutive days (pretreatment, cocaine conditioning). In the next 5 days, all animals rested without the drug (no-treatment, days 8-12). On day 13, half of the mice pretreated with vehicle and half of the mice pretreated with cocaine were treated i.p. with vehicle to evaluate conditioned locomotion (CL) response. Finally, on the last day (14), the remaining half of the mice pretreated with

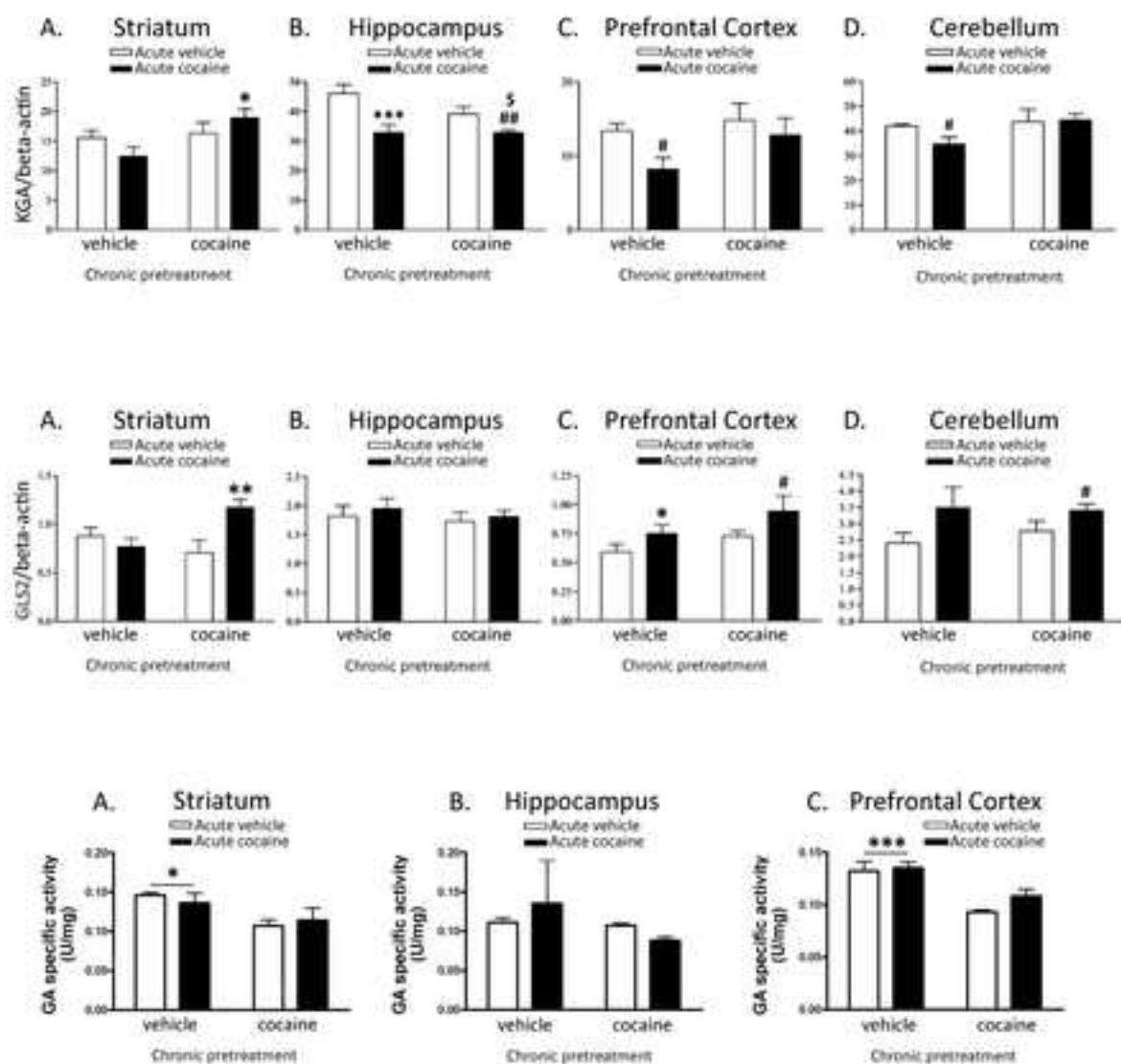
vehicle and the remaining half of the mice pretreated with cocaine were treated i.p. with a lower dose of cocaine (priming, 10 mg/Kg) to assess locomotor sensitization (LS) response. These independent groups of animals (chronic cocaine pretreatment + acute vehicle treatment, C-V group; chronic cocaine pretreatment + acute cocaine treatment, C-C group; chronic vehicle pretreatment + acute vehicle treatment, V-V group; and chronic vehicle pretreatment + acute cocaine treatment, V-C group) were employed by gene and enzymatic glutaminase activity studies. **Bottom panel:** quantitative real-time RT-PCR analysis of EAAC1 mRNA expression normalized to the levels of β -actin mRNA in different brain regions. Each value corresponds to mRNA levels in C57BL/6J mice after acute treatment (sensitization with vehicle or cocaine for 1 day) and chronic pretreatment (conditioning with vehicle or cocaine for 5 days). Bars indicate the mean value \pm SEM (n=7-8 per group). Two-way ANOVA, *p < 0.05 chronic pretreatment effect in cerebellum; **p < 0.01 acute treatment effect in striatum; **p < 0.01 chronic pretreatment effect in striatum (adapted from reference [63]).

Figure 3. Assessment of GA mRNA and enzymatic activity modifications caused by acute cocaine treatment (vehicle or cocaine for 1 day), chronic cocaine pretreatment (conditioning with vehicle or cocaine for 5 days) and its interaction (acute treatment x chronic pretreatment). **Top panel:** quantitative real-time RT-PCR analysis of KGA mRNA expression normalized to the levels of β -actin mRNA in different brain regions. Each value corresponds to mRNA levels in C57BL/6J mice after acute treatment (sensitization with vehicle or cocaine for 1 day) and chronic pretreatment (conditioning with vehicle or cocaine for 5 days). Bars indicate the mean value \pm SEM (n=7-8 per group). Two-way ANOVA: *p < 0.05 chronic pretreatment effect in striatum, ***p < 0.001 acute treatment in hippocampus; Bonferroni post hoc analysis: ##p < 0.01 vs. V-V group in hippocampus, #p < 0.05 vs. V-V group in PFC and cerebellum, ^sp < 0.05 vs. C-V group in hippocampus. **Medium panel:** quantitative real-time RT-PCR analysis of GLS2 mRNA expression normalized to the levels of β -actin mRNA in different brain regions. Each value corresponds to mRNA levels in C57BL/6J mice after acute treatment (sensitization with vehicle or cocaine for 1 day) and chronic pretreatment (conditioning with

1 vehicle or cocaine for 5 days). Bars indicate the mean value + SEM (n=7-8 per group). Two-
2 way ANOVA: **p<0.01 interaction (acute treatment x chronic pretreatment effect) in striatum,
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4 *p<0.05 acute treatment effect in PFC; Bonferroni post hoc analysis: #p<0.01 vs. V-V group in
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6 PFC and cerebellum. **Bottom panel:** quantification of total enzymatic activity of GA in striatum
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8 (a) (n=6), hippocampus (b) (n=6) and PFC (c) (n=6) after acute treatment vs. chronic
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10 pretreatment with vehicle and cocaine in mice. Two-way ANOVA: *p<0.05; ***p<0.001
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13 (adapted from references [63-66]).
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